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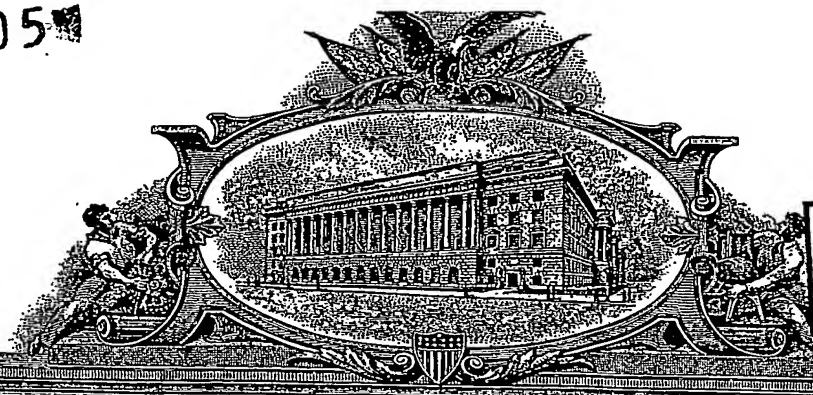

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APPLICATION NUMBER: *60/386,932*
FILING DATE: *June 06, 2002*
RELATED PCT APPLICATION NUMBER: *PCT/US03/17442*

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06/13/2002 VYANG1 00000015 60386932
01 FC:214 80.00 OP

PTO-1556
(5/87)

*U.S. Government Printing Office: 2001 — 481-697/59173

06-10-02 60386932.060602 ATRW

PTO/SB/16 (11-95)
Approved for use through 01/31/98. OMB 0651-0037
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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INVENTOR(s)/APPLICANT(s)

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TITLE OF THE INVENTION (280 characters max)

Novel Recombinant Anticoagulant Proteins

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ENCLOSED APPLICATION PARTS *(check all that apply)*

[X]	Specification	*Number of Pages*	38	[X]	Small Entity Statement
[X]	Drawing(s)	*Number of Sheets*	5	[ ]	Other (specify)

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Respectfully submitted,

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By: Tze-Chein Wun
Tze-Chein Wun

Date: June 6, 2002

Novel Recombinant Anticoagulant Proteins

Field of the Invention

The present invention relates to the field of blood coagulation inhibitors.

Background of the Invention

Tissue factor (TF) is generally considered to be the physiological trigger of the blood coagulation in normal hemostasis and in a variety of coagulopathic and thrombotic diseases. TF is an integral membrane protein that is normally present on the surface of certain extra-vascular cell types, but can also be induced to express on endothelium and monocytes upon stimulation [reviewed in (1)]. Based on studies in whole blood and re-constituted plasma systems (2-5), the key events of TF-initiated blood clotting can be schematically illustrated in Fig. 1. Upon exposure, TF forms a complex with factor VII/VIIa present in the circulating blood. The resulting extrinsic tenase (TF/VIIa) complex initiates the clotting cascade by activating small amounts of factors IX and X on the TF-bearing cells/microparticles. The TF/VIIa-activated factor IXa and Xa play distinct roles in the subsequent coagulation reactions. In a complex with factor Va/V on TF-bearing membrane surface, factor Xa generates a small amount of thrombin that partially activates platelets, cleaves fibrinogen to form an initial clot, and converts factors V, VIII, and XI to their active forms. Subsequent to this initiation phase, propagation of thrombin generation

begins. During the propagation phase, activated platelets provide an anionic membrane surface for the assembly of intrinsic tenase (VIIIa/IXa) and prothrombinase (Va/Xa) complexes, which very efficiently activate factor X and prothrombin, respectively, leading to explosive thrombin generation and consolidation of the fibrin-platelet plug.

Anionic phospholipid, chiefly phosphatidyl-L-serine (PS), is essential for assembly and expression of catalytic activities of the three membrane-associated enzymatic complexes (extrinsic tenase, intrinsic tenase and prothrombinase) that drives the initiation and propagation of the coagulation cascade. The plasma membrane phospholipids of mammalian cells are normally asymmetrically distributed with PS being exclusively sequestered in the inner membrane leaflet (6). As a consequence, intact quiescent cells are normally not procoagulant. In circumstances of cell activation, cell injury, or in response to apoptotic stimuli, phospholipid asymmetry across the plasma membrane collapses, resulting in exposure of PS on the membrane surface and shedding of membrane "microparticles". The exposure of PS allows assembly of enzyme/cofactor complexes and interaction with their substrates on the membrane surface, thereby greatly enhances the efficiency of the coagulation reactions (7-9). TF/VII(a) complex formed on intact cells are often cryptic in enzymatic activity towards its substrates. Many fold increase in TF/VIIa activity occurs when PS becomes available on the membrane surface after cell disruption, treatments with various agents, or induction of apoptosis (10-14). The rate of factor X activation by TF reconstituted with vesicles composed of phosphatidylcholine (PC) alone was less than 5 % of that observed with PS-PC vesicles (15). These observations suggest that concomitant expression of TF and exposure of PS on the membrane surface are

of key importance in the initiation of coagulation. One of the major roles of platelets in hemostasis/thrombosis is to provide an anionic membrane surface for the assembly of intrinsic tenase and prothrombinase (7,16). Upon activation, PS rapidly appears on platelet membrane surface. Interaction of factor VIIIa with the anionic lipid creates a Ca^{++}-dependent high-affinity binding site for factor IXa, leading to the formation of the intrinsic tenase complex. Likewise, binding of factor Va to the anionic lipid promotes Ca^{++}-dependent binding of factor Xa, forming the prothrombinase complex. Thus, availability of PS on the membrane surface is a key determinant that allows initiation and propagation of coagulation reactions to occur and be localized.

Tissue factor pathway inhibitor (TFPI) is a multivalent Kunitz-type inhibitor that regulates the tissue factor pathway of coagulation in the human vascular system (17). It inhibits factor Xa directly, and in a factor Xa-dependent manner, produces a feedback inhibition of TF/VIIa complex and thus dampens the protease cascade of the tissue factor pathway. Although TFPI is physiologically very important in the regulation of tissue factor pathway, its development for clinical antithrombotic therapy has been delayed due to large doses required to interrupt vascular thrombosis (18-20). Several naturally occurring Kunitz-type inhibitors that bind factors VIIa, IXa, Xa, and XIa of the tissue factor pathway have also been described. These include leech-derived Antistasin (ATS) (21), Tick Anticoagulant Peptide (TAP) (22), and two Ancylostoma caninum Anticoagulant Peptides (AcAP5 and AcAP6) (23) that inhibit factor Xa specifically; another Ancylostoma caninum Anticoagulant Peptide (AcAPc2) that inhibits VIIa (23); and a Kunitz-inhibitory domain of amyloid β-protein precursor (K_{APP}) that inhibits factors VIIa, IXa, Xa, and XIa (24-27).

Using site-specific mutagenesis and phage display technology, two series of K_{APP} and aprotinin (bovine pancreatic trypsin inhibitor) homologs with very high affinity (sub-nanomolar K_i) toward different coagulation proteases (TF/VIIa, Xa, XIa, and Kallikrein) have been created (28-31). Interestingly, the anticoagulant potencies of these mutants are quite low in *in vitro* coagulation assays (tissue factor-initiated clotting and activated partial thromboplastin time). The aprotinin homologs also require very high doses to achieve antithrombotic effect in an *in vivo* vascular trauma model (31).

Brief Description of the Invention

In accordance with the present invention novel recombinant anticoagulant proteins and method for their production are provided.

The blood coagulation cascade proceeds primarily via the formation of three procoagulant enzyme complexes, each consisting of a vitamin K-dependent serine protease associated with a membrane-bound cofactor on an anionic membrane surface. These complexes are conventionally named extrinsic tenase (factor VIIa-tissue factor), intrinsic tenase (factor IXa-factor VIIIa), and prothrombinase (factor Xa-factor Va). A novel series of recombinant anticoagulant fusion proteins are preferably created by linking annexin V (ANV), a phosphatidylserine binding protein, to Kunitz-type protease inhibitors (KPI) targeting the serine proteases in the enzymatic complexes. The resulting fusion proteins exhibit much stronger anticoagulant activities than their component proteins.

Several of these constructs possess far greater potencies than tissue factor pathway inhibitor, the natural inhibitor of tissue factor-induced coagulation in blood. The annexinV:Kunitz-type inhibitor fusions represent a new class of anticoagulants that specifically target the coagulation enzyme complexes on the procoagulant membrane surface, and are useful as anti-thrombotic therapeutic agents.

In brief, a series of unique recombinant fusions of annexin V (ANV) and Kunitz protease inhibitors (KPI) are thus preferably provided herein. For convenience, these recombinant anticoagulant fusion proteins are abbreviated ANV:KPI. These fusions utilize high affinities of ANV for phosphatidyl-L-serine (PS)(32) and various KPI for the serine proteases in membrane-associated coagulation complexes in the blood coagulation cascade.

Detailed Description of the Invention

The following detailed description of the invention taken in conjunction with the accompanying drawings is provided to further illustrate the invention and preferred embodiments in greater detail.

Brief Description of the Drawings

Fig. 1. Schematic of TF-initiated clotting.

Factor VII binds to TF and is activated to VIIa on the TF-bearing cells/microparticles.

The TF/VIIa complex activates both factor IX and factor X. The factor Xa generates a small amount of thrombin (IIa) locally. This small amount of thrombin activates platelets, activates factor V, releases factor VIII from von Willebrand factor and activates it, and activates factor XI. TF/VIIa-activated IXa can then bind to the VIIIa on the activated platelet to form an intrinsic tenase that activates factor X efficiently. The platelet-generated Xa binds Va to form a prothrombinase that promotes large-scale conversion of prothrombin (II) to thrombin. Adapted from Roberts et al. (5) and Mann K et al. (3).

Fig. 2. Schematic of annexin V and its fusion products with various Kunitz-type inhibitors.

ANV, annexin V; TAP-ANV, ala-tick anticoagulant peptide linked to annexin V by Gly-Ser dipeptide; ANV-6L15, annexin V linked to 6L15 (a Kunitz inhibitor with high affinity for TF/VIIa); ANV-K_{APP}, annexin V linked to K_{APP} (Kunitz inhibitory domain of amyloid β-protein precursor); ANV-KK_{TFPI}, annexin V linked to KK_{TFPI} (TFPI22-161 containing Kunitz-1 and Kunitz-2 domains).

Fig. 3. SDS-PAGE analysis of purified ANV and its fusion products with various Kunitz-type inhibitors.

Samples were analyzed by 12% SDS-PAGE under non-reducing (A) or reducing (B) conditions followed by coomassie blue staining. All samples were boiled for 3min without (A), or with (B) 50 mM dithiothreotol. Approximately 5 μg proteins were loaded on each lane. Lane 1, molecular weight marker; lane 2, ANV-KK_{TFPI}; lane 3, ANV-6L15; lane 4, TAP-ANV; lane 5 ANV-K_{APP}; lane 6, ANV.

Fig. 4. Inhibition of porcine trypsin and bovine factor Xa by various purified inhibitors.

Inhibitions of trypsin and bovine factor X were measured by amidolytic assays as described in Methods hereinbelow. The concentrations of active trypsin and bovine factor Xa were determined by active site titrations with 4-nitrophenyl *p'*-guanidinobenzoate (40,41). The concentrations of purified inhibitors were determined by absorbance measurement at 280nm using molar extinction coefficients of 28170, 7120, 39550, 18500, 31300, and 30170 for ANV-6L15, 6L15, TAP-ANV, TAP, ANV-K_{APP}, and ANV-KK_{TFPI}, respectively. (A) Inhibition of trypsin by ANV-6L15; (B) Inhibition of trypsin by 6L15; (C) Inhibition of factor Xa by TAP-ANV; (D) Inhibition of factor Xa by TAP; (E) Inhibition of trypsin by ANV-K_{APP}; (F) Inhibition of factor Xa by ANV-KK_{TFPI}.

Fig. 5. Effects of various inhibitors in activated partial thromboplastin time (APTT) assay.

APTT assay was carried out using an ACL 200 coagulometer and APTT-SP reagent (Instrument Laboratories). Pooled human plasma (180 µl) was mixed with 20µl of various inhibitors to attain the indicated final concentrations for the assay. The plasma with control buffer had a clotting time of 41 sec. TFPI (m) refers to mammalian C127 cell-derived FL-TFPI.

In order to further illustrate the invention, the following specific laboratory examples were carried out although it will be understood that the invention is not limited to these specific examples or the details described therein.

EXAMPLES

Materials and Methods

Reagents

Urea (sequenal grade) and Brij 35 were obtained from Pierce. Mixed bed resin AG501-X8, SDS-PAGE reagents, and molecular weight markers were purchased from Bio Rad. Dade Innovin® was from Baxter Diagnostics Inc. (Deerfield, IL). APTT-SP was from Instrumentation Laboratory (Lexington, MA). Bovine factor Xa was supplied by American Diagnostica, Inc. (Greenwich, CT). Trypsin, *p*-nitrophenyl *p'*-guanidinobenzoate HCl, bovine brain extract, cholesterol, and diacetylphosphate were from Sigma (St. Louis, MO). The synthetic substrates, S2444 and S2765 were obtained from diaPharma (West Chester, OH). Freshly frozen human plasma was purchased from Taipei Blood Center. Mammalian C127 cell- and *E. coli*- derived recombinant TFPI was prepared as described before (33,34). Recombinant X-K1 (C-terminal peptide of human factor X fused with the first Kunitz domain of TFPI) (35), and TFPI1-160 were gifts of Dr. George Broze, Jr., (Washington University). Yeast-derived recombinant TAP was a gift from Dr. Dana Abendschein (Washington University).

Cloning of cDNA for annexin V

ANV cDNA, lacking a stop codon, was generated from human placental mRNA by PCR using ANV reverse primer 1 (5'-ATCAAGCTTATGCATGTCATCTTC TCCACAGAG-3') and forward primer 2 (5'-GATCGGATCCAGTCTGGTCCTGCT TCACCTT -3'). ATGCAT is the site of restriction enzyme *Nsi* I used for ligating the K_{6L15}, K_{APP}, or $K1K2_{TFPI2-161}$ gene fragment. ANV cDNA mutation of Cys^{316}-to-Ala was created by PCR using oligonucleotide X (5'-CGTGACATGCATGTCATCTTCT CCAGCGAGCA-3'), in which the bolded GC was changed from CA in order to replace the original codon of Cys into Ala. Recombinant ANV was expressed without mutation of Cys^{316}. For all other ANV: KPI fusions, ANV cDNA with Cys^{316}-to-Ala mutation was used.

Construction of 6L15, TAP, and K_{APP} genes

The recombinant gene of K_{6L15} was assembled with 3 pairs of oligonucleotides through PCR technique. These are three forward primers of BP-1 (5'-TCCGGACTTCTGC CTGGAACCGCCGTACGACGGTCCGTGCCGTGCTCTGCACCTGCGTTACTTC-3'); BP-2 (5'-TACAATGCAAAGGCAGGCCTGTGTCAGACCTTCTACTACGGC GGTTGCCTGGCTAAGCGT-3'); BP-3 (5'-AACAACTTCGAATCCGCGGAACA CTGCATGCGTACTTGCGGTGGTGCTTA-3'); and three reverse primers of BP-1-3'(5'-ACGCAGGTGCAGAGCACGGCACGGACCGTCGTACGGCGGTTC CAGGCAGAAGTCCGGATGCAT-3'); BP-2-3' (5'-AGCCAGGCAACCGCCGTA GTAGAAGGTCTGACACAGGCCTGCCTTTGCATTGTAGAAGTA-3'); BP-3-3' (5'-AGCTTAAGCACCACCGCAAGTACGCATGCAGTCTTCCGCGGATTCGAA GTTGTTACGCTT-3'). The *Nsi*I restriction enzyme site ATGCAT was designed in the

primer sequence of BP-1-3' by changing original codon of Arg^1 into His for the ligating to the ANV gene fragment. For expression of 6L15, the original codon of Arg^1 was replaced by Ala.

The recombinant TAP and K_{APP} cDNA genes were each constructed by ligation of three pairs of overlapping synthesized oligonucleotides similar to that described above for 6L15.

The synthetic ala-TAP gene consists of the following sequence:

GCT TAC AAC CGT CTG TGC ATC AAA CCG CGT GAC TGG ATC GAC GAA TGC GAC TCC AAC GAA GGT GGT GAA CGT GCT TAC TTC CGT AAC GGT AAA GGT GGT TGC GAC TCC TTC TGG ATC TGC CCG GAA GAC CAC ACC GGT GCT GAC TAC TAC TCC TCC TAC CGT GAC TGC TTC AAC GCT TGC ATC TAA

The synthetic K_{APP} gene consists of the following sequence:

GAG GTT TGT TCT GAG CAA GCT GAG ACT GGT CCA TGT AGA GCT ATG ATT TCT AGA TGG TAC TTC GAC GTT ACT GAG GGT AAG TGT GCT CCA TTC TTC TAC GGT GGT TGT GGT GGT AAC AGA AAC AAC TTC GAC ACT GAG GAG TAC TGT ATG GCT GTT TGT GGT TCT GCT ATT TAA

Construction of E. coli expression plasmids

To construct the plasmids for expression of ANV-6L15 and ANV-KK_{TFPI} the following primers were used for PCR amplification and subcloning into pET20b expression vector:

ANV-nde (5'- G GAATTCCATATGGCACAGGTTCTCAGAGG-3'),

ANV-nsi (5'-CCAATGCATGTCATCTTCTCCAGC-3'),

K_{6l15}-nsi (5'-CCAATGCATCCGGACTTCTGCCTG-3'),

$K1K2_{TFPI}$-nsi (5'-CCAATGCATTCATTTTGTGCATTC-3'),

K_{6L15}-sal (5'-ACGCGTCGACTTA AGCACCACCGCAAG -3'), and

$K1K2_{TFPI}$-sal (5'- ACGCGTCGACTTAGGTTCCATA ATTATCC -3'). The sequence underlined is a *Nde* I restriction enzyme site and boxed is the cutting site for *Sal* I. The underlined ATGCAT is Nsi I restriction enzyme site used for gene fusion. The enlarged ATG is the initiation codon of Met and the TTA is a complimentary sequence to the stop codon of TAA. The PCR amplified gene fragment of ANV was digested with *Nde*I and *Nsi*I restriction enzymes and linked to *Nsi*I and *Sal*I digested 6L15 (or KK_{TFPI}) PCR fragment. The fusion gene was ligated into the expression vector of pET20b(+) which was linearized with *Nde*I and *Sal* I restriction enzymes.

To construct the plasmid for expression of TAP-ANV, the following primers were used for gene fusion and subcloning into pET20b:

TAP-nde (5'- GGAATTCCATATGGCTTACAACCGTCTGTG -3');

TAP-bam (5'- CGGGATCCGATGCAAGCGTTGAAGCAG -3');

ANV-bam (5'- CGGGATCCGCACAGGTTCTCAGAGGC -3');

ANV-sal (5'- ACGCGTCGACTTAGTCATCTTCTCCAGCG -3'). The PCR

amplified gene fragment of TAP was digested with *Nde*I and *Bam*HI restriction enzymes and linked to *Bam*HI and *Sal*I digested ANV gene fragment. The fusion gene was inserted into the expression vector of pET20b(+) which was also linearized with *Nde*I and *Sal* I restriction enzymes.

The desired recombinant plasmids were screened by PCR and DNA sequence determination. The expression plasmids are designated pET20b-AB8, pET20b-AKK11, and pET20b-TAP-A, which expressed intracellularly the recombinant proteins of ANV-6L15 and ANV-KK_{TFPI}, and TAP-ANV, respectively, in *E. coli* under the control of T7 promoter.

To express ANV and 6L15 for the purpose of comparison, the PCR-generated gene fragments of ANV and 6L15, respectively, were inserted into the plasmid using the same strategy for *E. coli* expression.

E. coli expression

E. coli BL21 (DE3) pLysS [(F *ompT* *hsd*S_B (r_B^-,m_B^-) *gal dcm* (DE3) pLysS $(Cam)^R$)] (Novagene, Madison, WI) was used for expression of recombinant proteins. *E. coli* DH5α [(F (ϕ80d *lacZ*ΔM15) Δ(*lacZYA-argF*)U169 *endA*1 *recA*1 *hsd*R17(r_K^- m_K^+) *deo*R *thi*-1 *sup*E44 *gyr*A96 *rel*A1 λ$^-$)] was used for construction of expression plasmids. The expression plasmid was propagated and isolated from *E. coli* DH5α and was transformed into the frozen competent cells of *E. coli* BL21. A single colony was inoculated into a 25-ml LB broth (containing 100 mg/L ampicillin and 34 mg/L chloroamphenicol), and

grown overnight at 37° with vigorous shaking. Ten ml of the overnight culture was inoculated into 1 liter of the same medium in a 2.8L flask (Nalgene) and maintained at 37° until the OD_{600} of the culture reached 0.5. The culture was induced by adding IPTG (Promega) to a final concentration of 1 mM and continuously shaking at 37° for 4 hours. The *E. coli* cells were harvested by centrifugation at 7000 rpm for 12 min. The cell pellet was frozen at –80° for further use.

Construction of Yeast expression plasmid

The *Pichia* expression vector of pPIC9, utilizes the strong and highly inducible P_{AOX1} promoter and α-factor signal peptide for high level expression and secretion of target proteins. The fragment containing the gene of interest was cloned in frame with the secretion signal peptide flanked by *Xho* I and *Not* I sites, the sequences from the *Xho* I site to the initiation codon of the target gene encoding the protease site of KEX2 must be created for occurrence of efficient cleavage of the fusion protein. The primers designed for generating PCR fragment of interest for cloning into vector pPIC9 were ANV-xho (5'-CCG *CTCGAG* AAA AGA GCA CAG GTT CTC AGA G-3'), K_{APP}-not (5'-ATA AGA AT GCGGCCGC TTA AAT AGC AGA ACC AC-3'), ANV-ecov (5'-CGC GAT ATC ATC TTC TCC AGC GAG-3'), 5'-K_{APP} (5'-GAG GTT TGT TCT GAG CAA GC-3'). The sequences *CTCGAG* and GCGGCCGC are *Xho* I and *Not* I restriction enzyme sites, respectively, used for editing the gene fragment and ligating into the vector pPIC9. The *CTCGAG* AAA AGA encoded 4 amino acids, Leu-Glu-Lys-Arg, which is a typical cleavage site for KEX 2 protease, so the following codon in the primer was designed to be the first codon (shown in enlarged text) of the secreted protein of interest.

For generating the ANV-K_{APP} fusion gene, we designed primer ANV-ecov which would create *Eco*RV site (GATATC) located at 3'-end of the ANV gene fragment without changing the last encoded amino acid (Asp). 5'-K_{APP} primer is a forward sequence of K_{APP} gene from the initiation codon of Glu (GAG). The K_{APP} gene fragment amplified by primers 5'-K_{APP} and K_{APP}-not was blunt-end ligated to ANV gene amplified by primers ANV-xho and ANV-ecov and digested by *Eco*RV to generate the fusion gene of ANV-K_{APP}. The fusion gene was digested by *Xho* I and *Not* I restriction enzymes and ligated to the pPIC9, which was linearized using the same enzymes. The ligation mixture was transformed into *E. coli* DH5α and the desired clone was screened by PCR and confirmed by DNA sequence analysis to identify the in frame amino acid sequence along with α-factor signal peptide. The resulting plasmid was pPIC9 ANV-K_{APP}.

Pichia expression

The yeast expression plasmid was propagated and isolated from *E. coli* DH5α. Integration was targeted by digesting the expression plasmid with *Sac* I restriction enzyme prior to transformation. The α-factor fused gene cassette including *His*4 as the selection marker was inserted into the genome of *P. pastoris* GS115 (*his*4) at the *AOX*1 locus via electroporation (36). The recombinant strains were selected by growing from the MD (minimal dextrose medium, 1.34 % yeast nitrogen base without amino acid-$4x10^{-5}$ % biotin-2 % dextrose-1.5 % bacto-agar) plate through the *His*4 compensation.

A single colony of *P. pastoris* GS115 recombinant strain from the MD plate was inoculated into 2 ml of BMGY medium (buffered glycerol complex medium, 1 % yeast extract-2 %

peptone-100 mM potassium phosphate, pH 6.0-1.34 % yeast nitrogen base without amino acid-4x10^{-5} % biotin-1 % glycerol) in 10 cm long Pyrex tube and grown at 30° with vigorous shaking at 200 rpm overnight until the OD_{600} of the culture reached 2-6. One ml of culture was harvested by centrifugation and resuspended into 3 ml of BMMY medium (buffered methanol complex medium, 1 % yeast extract-2 % peptone-100 mM potassium phosphate, pH 6.0-1.34 % yeast nitrogen base without amino acid-4x10^{-5} % biotin-0.5 % methanol) in 15 cm long Pyrex tube. The culture was maintained at 30° with vigorous shaking at 200 rpm for 24 hours for expression of the secreted protein. The cells were concentrated by centrifugation at 12,000 rpm for 10 minutes and the supernatant was assayed for inhibitory activity against trypsin. Ten µl of the supernatant was subjected to 12 % SDS-PAGE and the expressed ANV-K_{APP} was detected by Western blot.

For large-Scale Expression of ANV-K_{APP} in *Pichia*, a single colony of *P. pastoris* GS115 recombinant strain from the fresh MD plate was inoculated into 25 ml BMGY medium in a 300-ml flask and was grown at 30° with vigorous shaking at 200 rpm for 2 days. This late log phase culture was used to inoculate 400 ml fresh BMGY medium to a final OD_{600} of 0.1 in 1 L flask. The culture was maintained at 30° until OD_{600} reached 2. The cells were collected by centrifugation at 3000 rpm for 10 minutes in sterilized bottles and resuspended into 1 L of BMMY medium and transferred into 2.8 L flask. The culture was maintained at 30° with shaking to start induction of protein. After 24 hours of induction, the cells were removed by centrifugation and the supernatant was frozen at –80°.

Isolation of inclusion bodies from E.coli

Frozen *E. coli* cell paste was resuspended in cold Milli-Q water at a concentration of 75mg/ml. The cells were dispersed with a homogenizer for 30min on ice. The cells were then mechanically lysed by sonication. Lysate was centrifuged at 16,000g for 20min. The supernatant was discarded. The inclusion body pellets were collected, resuspended in the same volume of cold Milli-Q water, homogenized, sonicated, and pelleted by centrifugation as above one more time. The inclusion bodies were stored at -80^{0}.

Sulfonation of inclusion bodies and anion exchange chromatography

The buffers used for sulfonation, anion exchange chromatography, and protein refolding contained high concentration of urea. Urea solutions were treated with Bio Rad mixed bed resin AG®501-X8 at room temperature for at least 20 min and filtered through 0.2 μm filter before mixing with buffers. One gram of inclusion bodies (wet weight) was dispersed in 40ml of a solution containing 50mM Tris/HCl, pH 8.0, and 7.5 M urea by homogenization and vortexing. After the inclusion bodies were largely dissolved, 800mg of sodium sulfide was added, and the mixture was shaken at room temperature for 30min. Then, 400 mg of sodium tetracyanate was added and the mixture was shaken at 4^{0} overnight. The solution was dialyzed against 400ml of a solution containing 20mM Tris/HCl, pH 8, and 4 M urea. The dialyzed solution was centrifuged at 48,000xg for 1h, filtered through a 0.2 mm filter, and stored at -80^{0}. For anion exchange chromatography, 40 ml of sulfonated and dialyzed sample was loaded onto a HiLoad Q-Sepaharose 16/10 column pre-equilibrated in Q-buffer (20 mM Tris/HCl, pH 8-6 M urea-0.01% Brij 35) containing 0.15 M NaCl at room temperature. The column was washed with 240ml of equilibration

buffer and then eluted with a 396-ml gradient (0.15-0.4 M NaCl) in Q-buffer at a flow rate of 3 ml/min. Nine ml fractions were collected. The peak fractions containing the wanted protein was analyzed by SDS-PAGE, pooled, and used for refolding.

Refold of disulfide-containing proteins

A standard refold condition developed for refolding of *E. coli*-derived TFPI as described previously (34) was used for refolding of Kunitz inhibitors and ANV:KPI fusion proteins. In brief, the sulfonated and anion exchange chromatography pool was diluted to an absorbance of 0.07 at 280nm with Q-buffer containing 0.3 M NaCl. Solid L-cysteine was added to final concentration of 2 mM. The solution was incubated at room temperature for 24 h, diluted 1:1 with water with addition of 1 mM L-cysteine, and incubated at room temperature for another 24-48 h. For single-domain Kunitz proteins, refold can be carried out at a higher protein concentration (absorbance of 0.15 at 280nm) with essentially the same results.

Purification of 6L15 and ANV-KK$_{TFPI}$

Refold mixture of 6L15 (18ml) was acidified to pH 3.0 by titrating with 1 M citric acid, diluted 1:1 with water, and passed thorough a 1x8cm Q-Sepharose (fast flow) column pre-equilibrated in 20 mM Na-citrate, pH 3.0. The column was then eluted with a gradient from 0.1 to 1 M NaCl in the same buffer. 6L15 was eluted as a symmetrical peak around 0.5 M NaCl.

Refold mixture of ANV-KK$_{TFPI}$(600ml) was diluted 1:1 with water and passed through a

1x8cm Q-Sepharose (fast flow) pre-equilibrated with 5 mM Tris, pH 8.0-75 mM NaCl. The column was washed with 50ml of the equilibration buffer. ANV-KK_{TFPI} was then eluted with 5 mM Tris, pH 8.0-0.25 M NaCl.

Purification of TAP-ANV, ANV-6L15, ANV, and ANV-K_{APP}.

Refold mixture of TAP-ANV (160ml) was passed though a 1x8cm Q-Sepharose (fast flow) column pre-equilibrated in 20 mM Tris, 7.4. The column was washed with 50ml of the same buffer containing 0.15 M NaCl, then eluted with a gradient from 0.15 M to 0.35 M NaCl in the same buffer. TAP-ANV was eluted as a single symmetrical peak around 0.33 M NaCl. Refold mixture of ANV-6L15 was loaded on a 1x8cm Q-Sepharose (fast flow) column pre-equilibrated in 6.7 mM Tris, pH 9.5-2 M urea-0.003 % Brij35-0.1 M NaCl. The column was washed with 40ml of the same buffer, followed by 30ml of 20mM Tris, pH 7.4, then eluted with a 180ml-gradient from 0.1 M to 1 M NaCl in 20 mM Tris, pH 7.4. ANV-6L15 was eluted at around 0.28 M NaCl. The Q-Sepharose purified TAP-ANV and ANV-6L15 were further purified by adsorption to PS-containing liposomes by modification of the method described by Thiagarajan and Benedict (37). Multilamellar liposomes were prepared according to the method of Kinsky (38). Bovine brain extract (100 mg) containing 50% PS, 150mg cholesterol, and 10 mg diacetylphosphosphate were dissolved in chloroform and dried in a stream of nitrogen in a 40-ml glass vial. TBS (10 ml) was added to the vial and agitated vigorously in a vortex mixer for 5 min. The liposome was pelleted by centrifugation at 10,000g for 10min. The Q-Sepharose-purified TAP-ANV or ANV-6L15 was added to the liposome and $CaCl_2$ was added to a final concentration of 5 mM. The mixture was incubated at room temperature for 40 min, and then centrifuged at

10,000g for 10min. The pellet was washed with TBS-5 mM $CaCl_2$ four times by repeating centrifugation and re-suspension cycle as above. TAP-ANV or ANV-6L15 was eluted from the liposome using a solution containing 10 mM Tris, pH8.0-5 mM EDTA.

Recombinant ANV was directly isolated from *E. coli* lysate by binding to liposomes as described before (37) with some modification. In brief, the *E. coli* pellet was suspended in 50 mM Tris, pH 7.4-10 mM EDTA, and sonicated on ice to obtain lysate. The lysate was stored at –80°. Aliquot of the lysate was thawed, dialyzed against TBS, and clarified by centrifugation at 15,000g for 30min. The lysate was incubated with liposome in the presence of 5 mM $CaCl_2$ for 40min, followed by washing, centrifugation, and EDTA-elution as described above.

Recombinant ANV-K_{APP} was expressed and secrete into the culture medium of *pichia*. The medium was concentrated about 10 fold, exchanged with a buffer containing 10 mM Tris, pH 7.4-0.15 M NaCl, and clarified by centrifugation at 40,000g for 1h. The medium concentrate was incubated with liposome in the presence of 5 mM $CaCl_2$, followed by washing, centrifugation, and EDTA-elution as described above.

All the proteins eluted from liposomes by EDTA solution were subjected to centrifugation at 20,000g for 1h to separate the proteins from majority of the liposomes. To remove residual vesicles, the protein solutions were further filtered through CentriPlus YM-100 (Amicon).

Protein determination

The concentrations of proteins were determined by absorbance at 280nm using theoretical extinction coefficients calculated from amino acid sequence data as described by Gill and von Hippel (39). The following molar extinction coefficients were used: ANV (21,050); TAP-ANV (39,550); ANV-6L15 (28,170); ANV-K_{APP} (31,300); ANV-KK_{TFPI} (30,170); TAP (18,500); 6L15 (7,120); C127- and *E. coli*-derived FL-TFPI (20,650); C127 truncated TFPI (19,370); TFPI1-160 (7,840); X-K1 (14,490).

Amidolytic assays of trypsin and factor Xa inhibitory activities: Determination of stoichiometries of inhibitor-protease interactions

Bovine factor Xa (from American Diagnostica) and porcine trypsin (from Sigma) were titrated with *p*-nitrophenyl *p'*-guandininobenzoate according to Smith (40) and Chase and Shaw (41), respectively, to determine the concentrations of active factor Xa and trypsin. Inhibitory activities of TAP, TAP-ANV, and ANV-KK_{TFPI} against factor Xa were assayed by amidolysis of S2765. Ten µl of 50 nM bovine factor Xa in DB-buffer (10 mM Tris, pH7.5-0.15 M NaCl-1mg/ml BSA-0.002% Tween 20-0.02 % NaN_3) was mixed with 10 µl inhibitors diluted in the same buffer. After incubation at room temperature for 30 min, 10 µl of the reaction mixture was taken into 96-well plate and mixed with 85 µl of TBS-buffer (50mM Tris, pH 7.5-0.15 M NaCl-0.02% NaN_3) containing 5 mM $CaCl_2$. The absorbance change at 405nm was recorded on SPECTRAmax® PLUS^{384} (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer at room temperature for 60 sec. Stock solution of porcine trypsin was prepared in 50% glycerol-1 mM HCl-20 mM $CaCl_2$ and stored at -20^0. Inhibitory activities of 6L15, ANV-6-L15, and ANV-K_{APP} against trypsin were assayed by

amidolysis of S2444. A diluted trypsin solution (23nM) was freshly prepared from the stock in a buffer containing TBS-0.1 mg/ml BSA-20 mM $CaCl_2$. Ten ul of the trypsin solution was mixed with 10 µl of inhibitors diluted in the same buffer in the microplate well. After incubation at room temperature for 10min, 75 µl of TBS-20 mM $CaCl_2$ and 5µl of 10mM S2444 was added to the mixture and the absorbance change at 405nm was recorded on the microplate spectrophotometer at room temperature for 2min. In both assays, the fractional activities in the presence of inhibitors were calculated as percentages of that in the absence of inhibitors.

Plasma clotting time assays

Human plasma clotting assays were carried out on an ACL 200 coagulation analyzer (Instrumentation Laboratory, Lexington MA). A pooled normal plasma from 4 donors was used. For tissue factor-induced plasma clotting assay, each sample contains 100 µl of pooled plasma mixed with equal volume of an inhibitor dissolved in DB-buffer (10 mM Tris, pH 7.4-0.15 M NaCl-1 mg/ml BSA- 0.02% NaN_3) at varying concentrations. Inhibitor concentrations were calculated as nanomolar in plasma alone, not final plasma-buffer mixture. Innovin® (recombinant human tissue factor reconstituted with synthetic phospholipids) was diluted 1:100 with PT-buffer (75 mM NaCl-12.5 mM $CaCl_2$-0.5 mg/ml BSA-0.02 % NaN_3) for the assay. For activated partial thromboplastin time (APTT) assay, each sample contains 180 µl of pooled plasma mixed with 20µl of an inhibitor dissolved in DB-buffer at varying concentrations. Inhibitor concentrations were calculated as the final concentration in the plasma-buffer mixtures. APTT-SP reagent (Instrumentation Laboratory) was used for the assay without dilution.

Results

Construction and expression of recombinant ANV and ANV:KPI fusions

Plasmid vectors were constructed and used for expression of recombinant ANV and its fusion proteins with various Kunitz-type inhibitors possessing specific inhibitory activities against four key coagulation enzymes, factor VIIa, factor IXa, factor Xa, and factor XIa in the clotting cascade. Fig. 2. schematically depicts the molecular structures of these proteins. ANV was expressed as a full-length un-mutated molecule in *E. coli*. For other ANV-KPI fusions, the Cys^{316} of ANV was mutated to Ala to avoid forming disulfide bonds with the cysteines within the Kunitz domains during protein refolding. The fusion protein of TAP-ANV is total of 382 amino acid residues starting with an Ala residue followed by a 60 amino acids of TAP from Tyr^{1} to Ile^{60}, a dipeptide Gly-Ser, and a 319 amino acids of ANV(Cys^{316}-to-Ala). The fusion protein of ANV-K_{6L15} is total of 379 amino acid residues with a 319 amino acids of ANV(Cys^{316}-to-Ala) from initial Ala to the final amino acid (Asp) followed by a 60 amino acids of K_{6L15} from Met^{1} to Ala^{60}. In order to create the *Nsi*I restriction enzyme site for gene editing and ligation, the second amino acid of K_{6L15} in the fusion protein was changed from Arg to His. The fusion protein of ANV-K_{APP} is a polypeptide of 376 amino acids in total length. The N-terminus is the full length ANV(Cys^{316}-to-Ala) and the C-terminus is a 57 amino acids of K_{APP} polypeptide from Asp^{1} to Ile^{57}. The fusion protein of ANV-KK_{TFPI} is a polypeptide of 459 amino acid residues in length. The N-terminus of this fusion protein is a full length ANV(Cys^{316}-to-Ala) fused with a 140 amino acid polypeptide starting from Met^{22} to Thr^{161} of TFPI protein, including Kunitz domain 1 and 2.

Purification ANV and ANV:KPI fusion proteins

Recombinant ANV, TAP-ANV, ANV-6L15, and ANV-KK_{TFPI} were expressed intracellularly in *E. coli.* Essentially all the ANV molecules present in the *E. coli* lysate were capable of binding to PS-containing liposomes in the presence of Ca^{++} when analyzed by SDS-PAGE, suggesting that the expressed protein spontaneously folded itself into active forms. For other *E.coli*-expressed ANV:KPI fusions, majority of the expressed proteins occurred in inclusion bodies and required refolding to obtain active molecules. Using a sulfonation refold process developed previously for TFPI (34), we were able to achieve refolding of ANV:KPI fusion proteins as evidenced from the increase in inhibitory activity against trypsin or factor Xa during refolding. One-step Q-Sepharose chromatography of a refold mixture achieved high degree of purification as a single major band with the expected apparent molecular mass was observed in SDS-PAGE analysis. Further purification was carried out by binding to PS-containing liposomes in the presence of Ca^{++} followed by elution with EDTA. Recombinant ANV-K_{APP} was expressed and secreted into the culture medium of *P. pastoris* in active form. Active ANV-K_{APP} can be purified from concentrated medium by binding to PS-containing liposome in the presence of Ca^{++} and elution with EDTA. SDS-PAGE analysis of the final purified products is shown in Fig. 3. Under non-reducing condition (Fig. 3A), a major band was seen in each preparation. ANV-KK_{TFPI} (lane 2) and ANV (lane 6) both contained traces of dimmers. Under reducing condition (Figure 3B), the dimers disappeared and the fusion protein bands migrated slightly slower possibly because of disruption of disulfide bonds and unfolding of the Kunitz domains.

Stoichiometries of the interaction of the purified inhibitors with trypsin or factor Xa

Fig. 4 shows the titrations of trypsin or factor Xa activities by the purified inhibitors. Except ANV-KK_{TFPI}, the purified fusion inhibitors (ANV-6L15, TAP-ANV, and ANV-K_{APP}) and the Kunitz inhibitors (6L15 and TAP) all inhibits trypsin or factor Xa with apparent stoichiometries of 1:1. These results indicated that all the purified inhibitors containing a single Kunitz domain were substantially pure and fully active. The extent of deviation from 1;1 stoichiometry observed near equimolar concentration of inhibitor and enzyme reflects the variation in affinity of the interactions. The affinities of ANV-6L15, 6L15, and ANV-K_{APP} for trypsin (Fig 4 A, B, and E) appear stronger and the association of TAP-ANV and TAP with factor Xa (Fig. 4C, and D) appear weaker. Evidence of weaker affinities of TAP-ANV and TAP with factor Xa are also inferred from time-dependent slow increases of amidolytic activity upon addition of substrate and buffer in the assay. ANV-KK_{TFPI} inhibited factor Xa with an apparent stoichiometry greater than 2:1 under the experimental condition (Fig. 4F). Since a 1:1 stoichiometry is expected theoretically, it is possible that the purified ANV-KK_{TFPI} may contain inactive misfolded species. Alternatively, separate TFPI-K2 domain has been reported to bind with considerably reduced affinity with factor Xa (42) such that correct stoichiometry cannot be determined under the experimental condition used.

Prolongation of tissue factor-initiated clotting time

Purified inhibitors were added to pooled human plasma at different concentrations and plasma clotting was initiated by adding a diluted thromboplastin reagent (1:100 dilution of Dade Innovin®). Innovin® is a commercial preparation of recombinant human TF

reconstituted with an optimized phospholipid mixtures. The assay reagent contains both TF and anionic phospholipid to allow initiation and propagation of the coagulation cascade, and is a simplified system mimicking plasma clotting in the presence of activated TF-bearing cells/microparticles and platelets. The clotting time of the pooled plasma with added control buffer was 40.7 sec. With increasing concentration of added inhibitors, the clotting time was progressively prolonged. The concentration of inhibitors prolonging the clotting time 1.5 fold (i.e. from 40.7 to 61.1 sec) can be determined from the concentration-clotting time curves. Table 1 shows the concentrations required to prolong clotting time 1.5 fold for various inhibitors and their relative potency ranking. Since TFPI is the most important physiological regulator of the tissue factor pathway of coagulation in blood, and mammalian cell-derived TFPI may resemble most the former, we have chosen recombinant C127 FL-TFPI as a reference standard for comparison. TAP-ANV, presumably targeting the prothrombinase, is 86-fold more potent compare to C127 FL-TFPI. ANV-6L15, designed to inhibit TF/VIIa, is 12 fold more potent than C127 FL-TFPI. ANV-K_{APP} (possibly targeting TF/VIIa, VIIIa/IXa, and Va/Xa), ANV-KK_{TFPI} (presumably inhibiting TF/VIIa and Va/Xa), *E. coli*-derived non-glycosylated TFPI (presumably inhibiting TF/VIIa/Xa), and X-$K1_{TFPI}$ hybrid (likely inhibiting TF/VIIa) are 6-7 fold more potent than C127 FL-TFPI. ANV alone is 2.4 fold more potent than C127 FL-TFPI. TAP has the same potency as C127 FL-TFPI. Kunitz inhibitors alone, as exemplified here by TFPI1-160 and 6L15, are 40- and 59-fold, respectively, less active than C127 FL-TFPI.

Effects of various inhibitors on APTT

APTT measures the intrinsic pathway activity. The effects of various inhibitors in prolonging APTT are shown in Fig. 5. For the purpose of comparison, ANV is chosen as a reference standard. The most potent molecule, TAP-ANV, is about an order of magnitude more potent than ANV. The effect is likely mediated through inhibition of prothrombinase. ANV-K_{APP} (presumably inhibiting XIa, VIIIa/IXa, and Va/Xa), ANV-KK_{TFPI} (presumably inhibiting Va/Xa), and ANV-6L15 (possibly inhibiting kallikrein and XIa) are several-fold more potent than ANV. The Kunitz inhibitors alone (6L15, TAP, and TFPI1-160) are very weak in prolonging APTT. Interestingly, glycosylated mammalian C127 FL-TFPI is about an order of magnitude more potent than non-glycosylate *E. coli*-derived TFPI (Fig 5), the order of potency being reversed vs. that of tissue factor-induced clotting (Table 1). These results suggest that there are significant differences between mammalian- and *E. coli*-derived TFPIs.

Table 1:

Effects of various inhibitors on tissue factor-induced clotting time in human plasma.

Inhibitor	[a][Inhibitor]$_{1.5xCT}$, (nM)	[b]Relative potency
TAP-ANV	0.80	86
ANV-6L15	6.0	12
ANV-K_{APP}	9.4	7.3
X-K1$_{TFPI}$	10	6.9
ANV-KK$_{TFPI22-160}$	11	6.3
E. coli ala-TFPI	19	6.3
ANV	29	2.4
TAP	68	1
C127 FL-TFPI[c]	69	1
C127 CT-TFPI[c]	1300	0.053
E. coli TFPI1-160	2750	0.025
6L15	5900	0.017

[a] [Inhibitor]$_{1.5CT}$ is the concentration of inhibitor that prolong the tissue factor-induced clotting time 1.5 fold relative to control (from 40.7 to 61.1sec) as determined from concentration-dependent clotting time curves for each inhibitor.

[b] Relative potency is calculated from [Inhibitor]$_{1.5CT}$ using mammalian C127 FL-TFPI as reference standard (assigning C127 FL-TFPI as 1).

[c] C127 FL-TFPI refers to full-length molecules; CT-TFPI refers to molecules truncated at the carboxyl terminus as described previously (33).

Although the inventor is not to be bound by theory, it is believed that the foregoing results can be explained and elaborated thereon as follows:

Formation of extrinsic tenase (TF/VIIa), intrinsic tenase (VIIIa/IXa), and prothrombinase (Va/Xa) enzymatic complexes on anionic membrane surfaces are the key processes by which initiation and propagation of the tissue factor pathway of coagulation occur. Three anticoagulant systems, TFPI, antithrombin, and protein C, counter balance these procoagulant reactions. TFPI influences the initiation phase; antithrombin III decreases thrombin generation and activity in the propagation phase; and activated protein C affects the duration of the propagation by inactivating factor Va. As a consequence of the opposing actions of these pro- and anti- coagulants, tissue factor pathway to thrombin generation manifests as a threshold-limited process (3). When a certain threshold is exceeded, full coagulation cascade occurs despite the presence of large molar excess of the anticoagulants. TFPI is the primary physiological regulator of the tissue factor pathway. TFPI does not directly inhibit TF/VIIa complex per se, but in stead, must await generation of factor Xa first before forming an inert quartnary TFPI/Xa /TF/VIIa complex (17). Generation of factor Xa leads to formation of prothrombinase (Va/Xa), and once formed, prothrombinase is protected from inactivation by physiological concentration of TFPI (43,44). During this process, some intrinsic tenase (VIIIa/IXa) is also generated which is resistant to inhibition by TFPI and antithrombin III. As a result, TFPI regulates tissue factor pathway in a rather "leaky" manner. In *in vitro* clotting assay using 1:100 dilution of a commercial thromboplastin reagent, it requires 69 nM of mammalian cell-derived full-length TFPI to prolong clotting time just 1.5 fold (Table 1). In *in vivo* thrombosis

models, efficacies were observed only when high concentrations of TFPI (100-200 nM) are present in circulating blood or topically (18,19,45). These therapeutic doses of TFPI represent about 100-200 fold of that in the normal plasma. The apparent low potency and the large infusion dose required to achieve the desired blood level make TFPI less than ideal for therapeutic applications. Hence, it is of great use to have alternative molecules that exert better control of the tissue factor pathway of coagulation.

Coagulation cascade reactions are localized on PS-exposed membrane surfaces that facilitate the assembly of the coagulation complexes and enhance the catalytic efficiency. In the present work, it is hypothesized that enzyme inhibitors that have been conferred the ability to target themselves to the membrane-associated PS would become site-specific and more effective in inhibiting the coagulation complexes. To test this hypothesis, recombinant DNA technology was used to create four fusion proteins that share a common ANV domain linking to different KPI domains (TAP, 6L15, K_{APP}, and $K1K2_{TFPI}$). The ANV moiety has high affinity (K_d <0.1 nM) for membranes containing PS (32). The four KPIs chosen for this study have the following inhibition constants (K_i) for various coagulation serine proteases: TAP (0.2 nM for Xa); 6L15 (0.2 nM for TF/VIIa, 0.02 nM for plasma kallikrein and 13 nM for XIa); K_{APP} (68 nM for TF_{219}/VIIa; 13 nM for Xa; 190 nM for IXa; and 0.01 nM for XIa.); and $K1K2_{TFPI}$ (90 nM for Xa and 240 nM for TF/VIIa) (22, 24-27, 30, 31, 42). In *in vitro* clotting assays, the KPIs all require fairly high concentrations in plasma to prolong clotting times (22, 30, 47, Table 1 and Figure 5). All the four ANV:KPI fusion proteins, in contrast, prolong the plasma clotting times at greatly reduced concentrations compared to their component ANV and KPIs (Table 1 and Figure

In both TF-induced plasma clotting and APTT assays, the most potent fusion protein is TAP-ANV. This molecule inhibits prothrombinase since TAP is a highly specific inhibitor of factor Xa. The result indicates that inhibition at the prothrombinase level of the cascade is more effective compared with inhibition at the levels of TF/VIIa and VIIIa/IXa. This is consistent with the notion that factor Xa and prothrombinase generation is the rate-limiting step in the coagulation cascade (3). It is significant to note that 6L15 is a very poor inhibitor of tissue factor-initiated plasma clotting (Table 1) in spite of its high affinity binding with TF/VIIa (K_i 0.2 nM) (30). Thus, high affinity binding of TF/VIIa alone does not correlate with good potency in inhibiting TF-initiated clotting cascade. In contrast, the fusion inhibitor of ANV-6L15 is about three-order of magnitude more potent than 6L15 in inhibiting TF-initiated clotting, indicating that binding to PS greatly facilitates the inhibition of TF/VIIa by 6L15. The four fusion molecules created herein all show higher anticoagulant activities than mammalian cell-derived TFPI as assessed by TF-initiated plasma clotting and APTT assays. Thus. these molecules are superior to natural TFPI as anticoagulant and antithrombotic therapeutic agents.

An important attribute of the fusion proteins described herein is the presence of ANV moiety that confers on them the property of binding specifically to PS with high affinity. This indicates that these molecules possess an intrinsic property of targeting themselves to sites of thrombus formation where PS becomes available for assembly of coagulation complexes. Owing to their ability to target thrombogenic sites, it is potentially feasible to achieve antithrombotic effect without maintaining high levels of these anticoagulants in systemic circulation, thereby minimizing risks of systemic bleeding side effect. *In vivo*

animal study has demonstrated that ANV can specifically target and accumulate on platelet-containing thrombi (48). Furthermore, ANV dose-dependently inhibit thrombus formation in arterial and venous thrombosis models (37,49,50). Since the ANV:KPI fusion proteins possess higher potencies, they are superior to ANV as antithrombotic agents.

As demonstrated herein, this series of recombinant proteins can be produced in *E. coli* and yeast. In the *E. coli* system, the proteins can be expressed at very high levels in the inclusion bodies, and active molecules can be obtained by simple refolding and purification procedures. In the *Pichia* system, the protein can be secreted into the culture medium in active form and purified by the same simple procedure. From a manufacturing standpoint, ease and low cost of production are of great advantage.

Based on the results of the present work, it is believed that other fusion molecules of similar conceptual design can be created. For example: fusions of ANV with other natural KPIs such as Antistasin and Acylostoma caninum anticoagulant peptides; homologs and mutants of KPIs; and small-molecule inhibitors of factors VIIa, IXa, Xa, and XIa. In another variation, other PS binding protein moieties such as C2 domain of factor V and phospholipase A_2 fragment can be used in place of ANV for the creation of fusion molecules.

All such other examples as will be apparent to the person skilled in the art after reading the present disclosure are intended to be included within the scope of the present invention.

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CLAIMS

1. Recombinant anticoagulant proteins comprising fusions of annexin V (ANV) and Kunitz protease inhibitors (KPI).

2. Recombinant anticoagulant proteins of Claim 1 selected from the group consisting of TAP-ANV, ANV-6L15, ANV-K_{APP} and ANV-$KK_{TFPI\ 22\text{-}160}$

ABSTRACT OF THE DISCLOSURE

The disclosure describes novel recombinant anticoagulation proteins and method for their production. In particular, a series on unique recombinant fusions of annexin V (ANV) and Kunitz protease inhibitors (KPI) that possess substantially stronger anticoagulant activities than their component proteins are provided. These fusions, abbreviated ANV:KPI, are also more potent than TFPI in the inhibition of tissue factor-initiated clotting of plasma. These fusions utilize high affinities of ANV for phosphatidyl-L-serine (PS) and various KPI for the serine proteases in membrane-associated coagulation complexes in the blood coagulation cascade.

*>PTO/SB/09 (6-95)
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Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE<

VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) & 1.27(b))--INDEPENDENT INVENTOR	Docket Number (Optional) T-Z W1

Applicant or Patentee: Tze-Chein Wun

*>Application< or Patent No.: _____

Filed or Issued: _____

Title: Novel Recombinant Anticoagulant Proteins

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees to the Patent and Trademark Office described in:

[X] the specification filed herewith with title as listed above.

[ ] the application identified above.

[ ] the patent identified above.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

[X] No such person, concern, or organization exists.

[ ] Each such person, concern or organization is listed below.

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Tze-Chein Wun		
NAME OF INVENTOR	NAME OF INVENTOR	NAME OF INVENTOR
Tzechein Wun		
Signature of inventor	Signature of inventor	Signature of inventor
June 6, 2002		
Date	Date	Date

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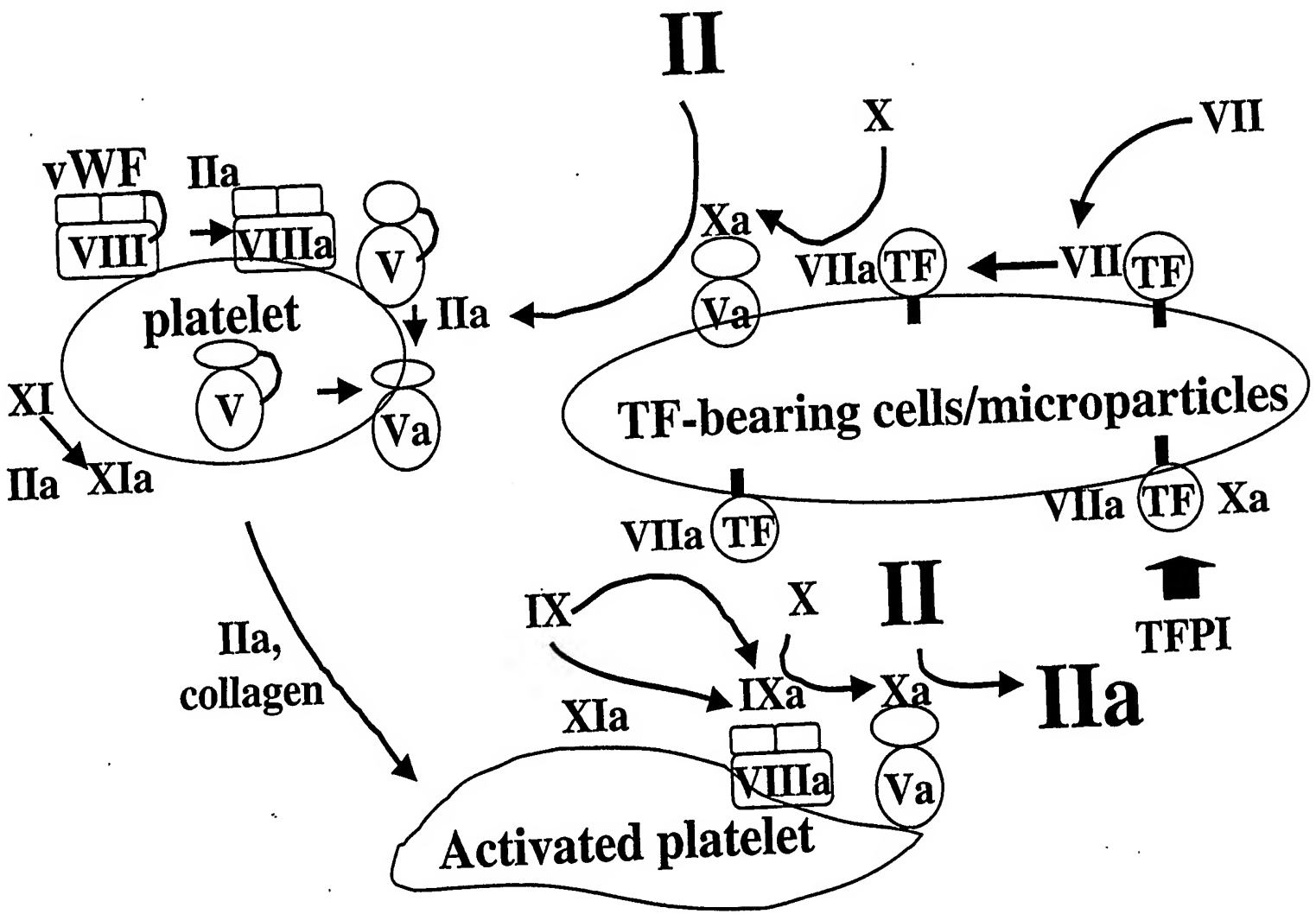
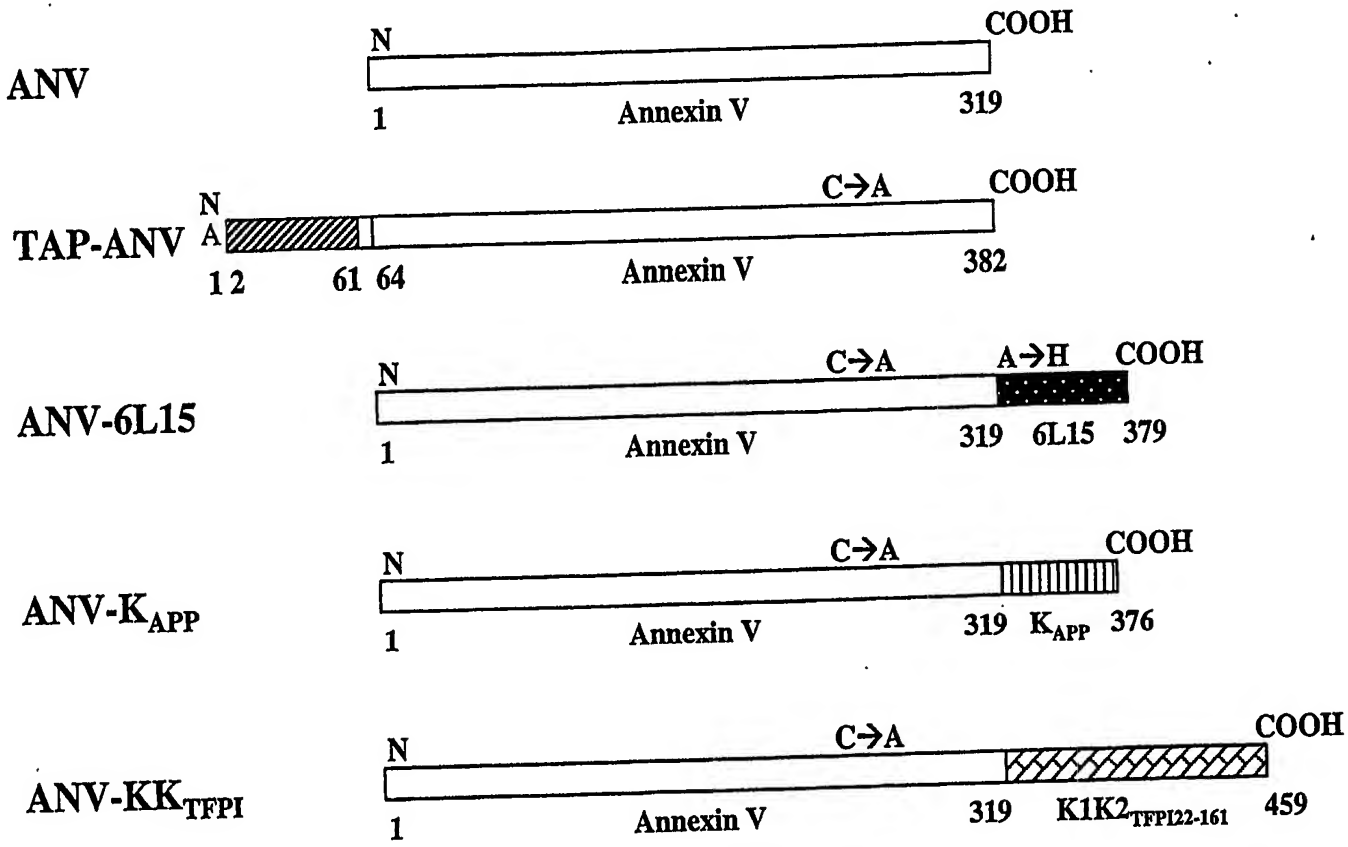


FIG. 1



F16.2

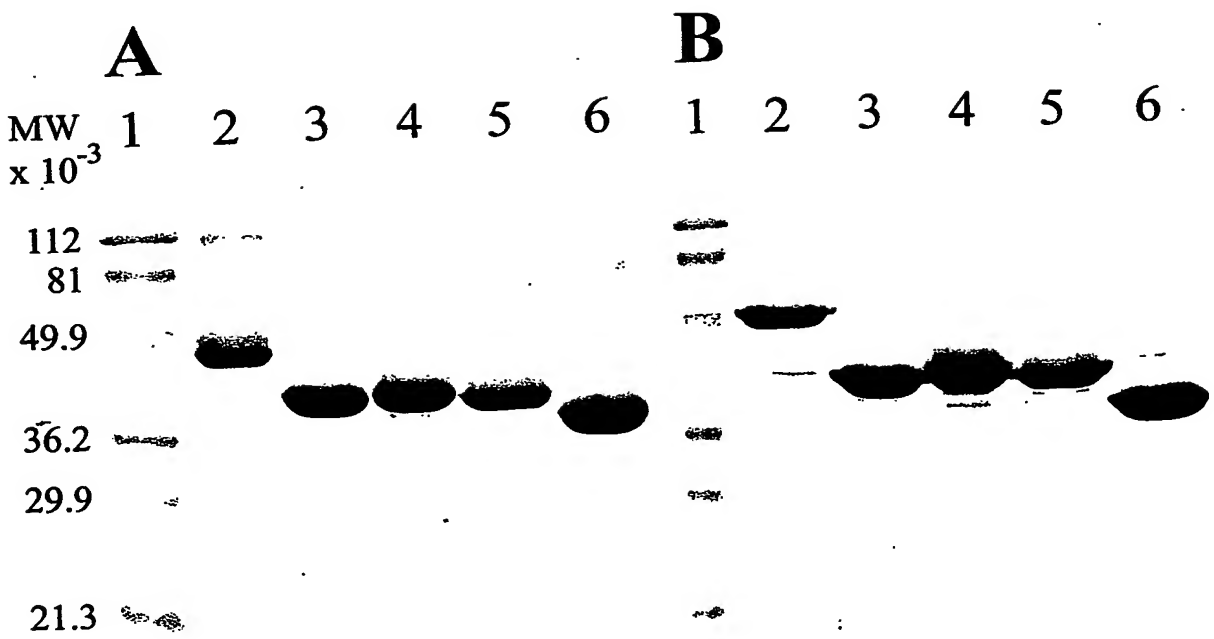


FIG. 3

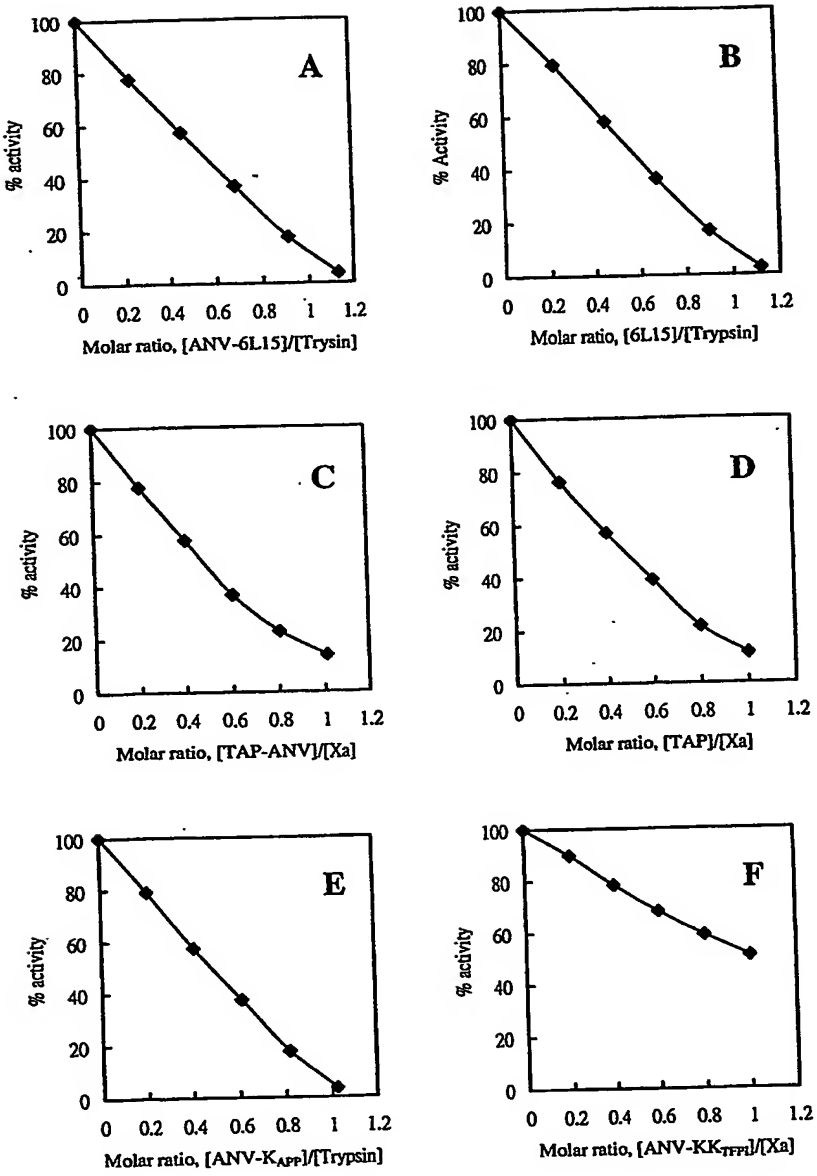

A
% activity
Molar ratio, [ANV-6L15]/[Trysin]
B
% Activity
Molar ratio, [6L15]/[Trypsin]
C
% activity
Molar ratio, [TAP-ANV]/[Xa]
D
% activity
Molar ratio, [TAP]/[Xa]
E
% activity
Molar ratio, [ANV-K$_{APP}$]/[Trypsin]
F
% activity
Molar ratio, [ANV-KK$_{TFPI}$]/[Xa]

FIG 9

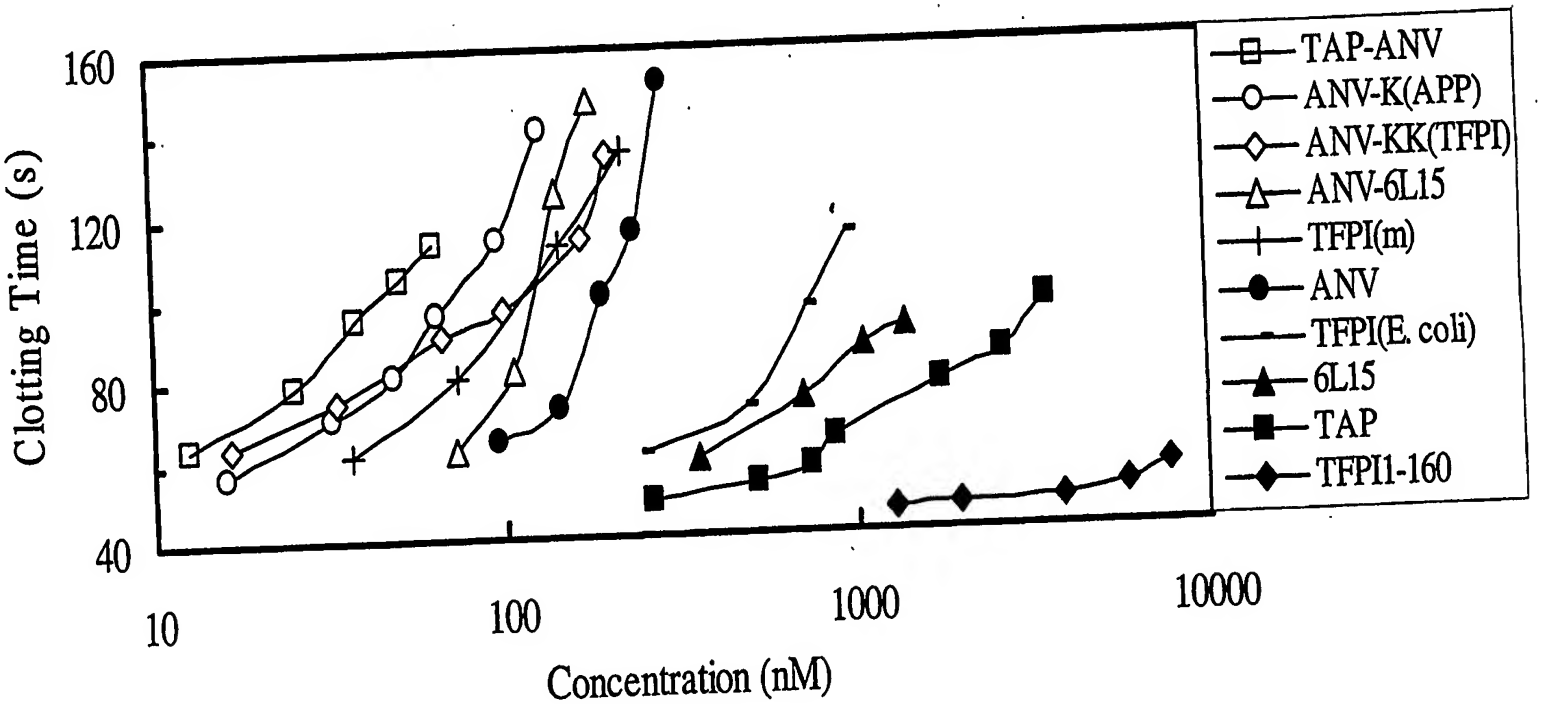

Clotting Time (s)
160
120
80
40
10
100
1000
10000
Concentration (nM)
TAP-ANV
ANV-K(APP)
ANV-KK(TFPI)
ANV-6L15
TFPI(m)
ANV
TFPI(E. coli)
6L15
TAP
TFPI1-160

FIG. 5